Global analysis of genomic instability caused by DNA replication stress in *Saccharomyces cerevisiae*

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DNA replication stress (DRS)-induced genomic instability is an important factor driving cancer development. To understand the mechanisms of DRS-associated genomic instability, we measured the rates of genomic alterations throughout the genome in a yeast strain with lowered expression of the replicative DNA polymerase δ. By a genetic test, we showed that most recombinogenic DNA lesions were introduced during S or G_2 phase, presumably as a consequence of broken replication forks. We observed a high rate of chromosome loss, likely reflecting a reduced capacity of the low-polymerase strains to repair double-stranded DNA breaks (DSBs). We also observed a high frequency of deletion events within tandemly repeated genes such as the ribosomal RNA genes. By whole-genome sequencing, we found that low levels of DNA polymerase δ elevated mutation rates, both single-base mutations and small insertions/deletions. Finally, we showed that cells with low levels of DNA polymerase δ tended to accumulate small promoter mutations that increased the expression of this polymerase. These deletions conferred a selective growth advantage to cells, demonstrating that DRS can be one factor driving phenotypic evolution.

DNA replication stress | DNA polymerase | genome instability

In normal rapidly dividing cells, DNA replication is rapid and accurate, preventing the accumulation of genomic alterations. Stalling of replication forks or inappropriate initiation of replication origins can result in DNA replication stress (DRS) that can contribute to cancer development (1). It has been proposed that mutations in oncogenes and tumor suppressor genes drive cell proliferation and induce DRS. In turn, DRS generates genome instability, allowing cells with various types of genetic variations (mutations, duplications, translocations) to escape cellular senescence and apoptosis (2). However, the mechanisms by which oncogenes induce DRS and the precise nature of DRS-associated DNA lesions have not been clearly defined.

Exposure of mammalian cells in culture to conditions that perturb DNA synthesis result in "fragile sites," gaps or constrictions detected by light microscopy in metaphase chromosomes (3). Aphidicolin, a drug that inhibits DNA polymerase, is one agent that induces fragile sites. The break points of chromosome rearrangements that occur in tumor cells often colocalize with fragile sites (3, 4), establishing another link between DRS and cancer. In addition to inducing chromosome breaks in cultured cells, aphidicolin induces high frequencies of duplications and deletions similar to those observed in tumor cells (5).

As a model for mammalian fragile sites, we previously constructed yeast strains in which the transcription of the replicative DNA polymerases α (encoded by *POL1*) or δ (encoded by *POL3*) was regulated by the *GAL1* promoter (6–8). Under low-galactose growth conditions, which reduced the levels of DNA polymerases α or δ about 10-fold, these strains had elevated rates of chromosome loss and rearrangements on chromosome III.

We also studied genome stability in diploid strains heterozygous for many (>50,000) single-nucleotide polymorphisms (SNPs) by using SNP-specific microarrays to detect loss of heterozygosity (LOH) and other genomic alterations (9). We applied this method to map events associated with low DNA polymerase α, and showed that LOH events were often associated with chromosome elements [such as quadruplex motifs, termination (ter) sequences, and long-terminal repeats (LTRs)] that slow replication forks (10).

In addition to the LOH and chromosomal rearrangements, single-base mutations and small insertions/deletions (in/dels) are also prevalent in some solid tumors (11). In our previous study (6), we found low levels of DNA polymerase-δ–induced mutations in the *CAN1* gene. However, the global mutagenic effects of reduced expression of DNA polymerase δ are unknown.

In the current study, by a combination of microarray analysis and whole-genome sequencing, we examine the effects of low levels of DNA polymerase δ on the rates of mitotic recombination, large (>1-kb) deletions and duplications, aneuploidy, single-base mutations, and small in/dels throughout the genome. We show that some of the observed alterations alleviate DRS. Our findings are relevant to the mechanism by which DRS drives genome instability, and the mechanism by which genetic alterations allow cells to escape DRS.

Results

As described below, we characterized genetic instability induced by low levels of DNA polymerase δ by two methods: SNP-specific

Significance

One important source of genomic instability associated with tumor cells is DNA replication stress. In the current study, replication stress was induced in yeast by a 10-fold reduction in the level of the replicative DNA polymerase δ. By DNA microarray analysis and high-throughput DNA sequencing, we showed that this stress resulted in very high rates of both large (aneuploidy, mitotic recombination, deletions and duplications, and translocations) and small (point mutations and small insertion/deletions) genetic alterations. Some of these changes resulted in a selective growth advantage of the cells, demonstrating the role of elevated genetic instability in the rapid evolution of cells in challenging growth conditions.

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The authors declare no conflict of interest.

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE78856). The sequencing data reported in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) (accession no. SRP071648).

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microarrays (allowing the mapping of mitotic crossovers, large deletions/duplications, and ploidy alterations) and DNA sequencing (allowing the detection of point mutations, small deletions/duplications, and changes in the copy number of repeated genes). Certain features of the instability are similar to those observed in strains with low levels of DNA polymerase α (for example, the association between recombination break points and hard-to-replicate genomic sequences), whereas others are quite different (for example, the ratio of ploidy alterations to mitotic recombination events, and elevated levels of point mutations).

System for Detecting Genomic Alterations by SNP-Specific Microarrays. We mapped LOH events and other chromosome alterations using SNP-specific microarrays as described previously (9). The diploid used in our experiment (DZ12) was constructed by crossing haploid strains of two sequence-diverged backgrounds, one isogenic with W303-1A (12) and one isogenic with YJM789 (13). These resulting diploid is heterozygous for about 55,000 SNPs. In DZ12, LOH events or ploidy alterations can be detected using SNP-specific microarrays. We designed arrays with 25-base oligonucleotides distributed throughout the genome that allow us to detect LOH at 13,000 positions. Each position is represented by four oligonucleotides, two with the Watson and Crick sequences of the W303-1A–specific allele and two with the Watson and Crick sequences of the YJM789-specific allele. By measuring the hybridization levels to each of these oligonucleotides (*Supporting Information*), we can detect LOH events and other alterations. Among the alterations detected in our experiments are gene conversion events (interstitial LOH events in which sequences from one homolog are duplicated and sequences from the other homolog are lost; Fig. 1*A*), crossovers (terminal LOH events in which sequences from one homolog are duplicated and sequences from the other are lost; Fig. 1*B*), large deletions (Fig. 1*C*) or duplications, and ploidy changes (Fig. 1*D*).

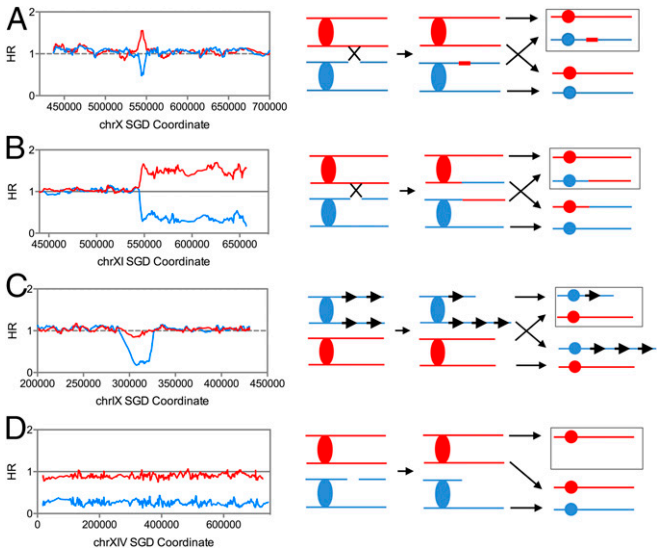

Fig. 1. Examples of LOH and other genomic rearrangements detected by microarray. (*A*) Microarray analysis of a gene conversion event on chromosome X in isolate DZ12-3. The *y* axis shows the normalized hybridization ratio between W303-1A–specific SNPs (red) and YJM789-specific SNPs (blue); the *x* axis shows SGD coordinates numbered from the left telomere. A moving window that includes the hybridization values of nine SNPs is shown. On the right side of the figure, the chromatids are shown as horizontal lines connected by ovals (centromeres). The daughter cell with the chromosomes represented by the microarray is outlined. (*B*) Microarray analysis of a crossover (or break-induced replication event) on chromosome XI in isolate DZ12-2. (*C*) A heterozygous deletion on chromosome IX in isolate DZ12-7. Because the break points of the deletion occur at repetitive δ elements (indicated as arrows on the right side of the figure), one possible mechanism of deletion formation is unequal crossing over. (*D*) Microarray analysis of an isolate monosomic for chromosome XIV (DZ12-2).

The DZ12 strain was also homozygous for an insertion of the *GAL1* promoter upstream of the coding sequence of *POL3*. When this strain was grown in low-galactose medium, the strain grew slowly and had an elongated S phase (Fig. S1). Under these conditions, the level of DNA polymerase δ is approximately 10-fold less than the level observed in a wild-type strain (6). When the strain is grown in high-galactose medium, the strain grew at approximately the same rate as a wild-type strain (Fig. S1*B*) and had a level of DNA polymerase δ that is about 7-fold higher than wild type (6). Last, we deleted the *MATα* locus from the diploid to prevent sporulation and to allow synchronization of the diploid using the α pheromone.

Characterization of LOH Events in DZ12. Single cells of strain DZ12 were allowed to form colonies on solid low-galactose medium. Because such colonies are likely to contain subpopulations of cells with different genotypes, we restreaked cells from 35 independent colonies to high-galactose medium in which the cells have much reduced rates of instability (6). Single colonies from the high-galactose plates derived from each of the original 35 colonies grown on low-galactose plates were subsequently analyzed by SNP-specific microarrays to detect LOH events and other chromosome alterations. Among the 35 isolates, we detected a total of 21 interstitial LOH events (gene conversions) and 69 terminal LOH events. There are two alternative mechanisms for generating terminal LOH events, crossovers and break-induced replication (BIR) events. In BIR events, a broken chromatid invades the other homolog and copies sequences from the point of invasion to the end of the chromosome (14). In wild-type diploid cells, DSBs are more frequently repaired by crossing over than BIR (15). Because the distinction between crossovers and BIR requires the recovery of both daughter cells, terminal LOH events in which sequences from one homolog are lost and sequences from the other homolog are duplicated will be described as "crossover/BIR" events.

The locations [*Saccharomyces* Genome Database (SGD) coordinates] of break points of LOH events are in Dataset S1. All LOH events were assigned a class (depicted in Dataset S2) depending on whether the event was a terminal or interstitial event, and which chromatid was the recipient of information. From previous studies of recombination (16), the chromosome with the recombinogenic DNA lesion acts as the recipient of sequences from the intact donor. In Fig. 1*A*, for example, we infer that the initiating DNA break was on the YJM789-derived homolog.

We also identified large interstitial deletions and duplications (Datasets S3.1 and S4). In these isolates, the hybridization signal for one homolog is reduced (deletion) or elevated (duplication), whereas the signal for the other homolog is unchanged. In Fig. 1*C*, we show a heterozygous deletion in which sequences were lost from the W303-1A–derived copy of chromosome IX in isolate DZ12-7. Of the 41 interstitial deletion/duplications detected in the 35 colonies, 21 were within the tandem cluster of *CUP1* genes, 10 involved homologous recombination between nonallelic Ty transposons, and 4 were between solo LTRs including the event shown in Fig. 1*C*. Six events were deletions between tandemly repeated *HXT* genes. In summary, the main source of large interstitial deletions and duplications is homologous recombination between ectopic repeats rather than nonhomologous end joining. Interstitial deletions outnumbered interstitial duplications 37 to 4.

Dataset S3.2 lists 6 terminal duplications and 10 terminal deletions. We found most (12 of 16) terminal alterations were paired events: a terminal duplication and a terminal deletion within one strain with break points that involved Ty elements

(Dataset S3.2). Fig. S2 shows one such event in which a region of chromosome IV was duplicated (Fig. S2*A*) and a region of chromosome VI was deleted (Fig. S2*B*). We previously found that a break in or near a Ty element on one chromosome could be repaired by break-induced replication using an ectopic Ty element on a nonhomologous chromosome as a template (Fig. S2*C*). The expected size of the translocated chromosome is about 655 kb, and a chromosome of this size was detected (Fig. S2*D*). The locations of the LOH events, and internal and terminal deletions/duplications are in Fig. 2*A*.

Analysis of Aneuploidy in DZ12. The same 35 colonies examined for LOH events had many changes in chromosome number. We observed 97 monosomic chromosomes and 8 trisomic chromosomes (Table S1). We also observed 11 uniparental disomy (UPD) events in which the diploid has two copies of the homolog, but both copies are derived from one of the two parental homologs. Two-way hierarchical clustering analysis of these aneuploidy events was performed using the R package "pheatmap." By a χ^2 test, monosomy for chromosomes V and XIV is significant ($P < 0.05$), elevated compared with the other homologs.

A summary of all genomic changes detected by microarrays is in Dataset S5. A small number of additional changes that were detected only by DNA sequencing are described below and in Dataset S6. Although we previously found elevated rates of LOH and ploidy changes in strains with low levels of DNA polymerase α (10), strains with low levels of DNA polymerase δ had a relatively higher ratio of ploidy alterations (Fig. S3).

One cycle of growth in low-galactose medium resulted in an average of 14 alterations/isolate and 8 alterations/isolate for the strains with low levels of DNA polymerase α and δ, respectively. Because it takes about 25 divisions to form a colony from a single cell, the rates of alterations per cell division in the strains with low levels of DNA polymerase α and δ are about 0.6 and 0.3, respectively. By a similar calculation using the data of O'Connell et al. (17), the rate of genetic alterations in wild-type cells is about 2.4×10^{-3} per division. Thus, low levels of either DNA polymerase α or δ elevate genomic instability by about two orders of magnitude. We also examined six isolates of DZ12 that were grown from a single cell to a colony on high-galactose medium. These isolates had an average of less than one genomic alteration per isolate. In summary, replication stress imposed by

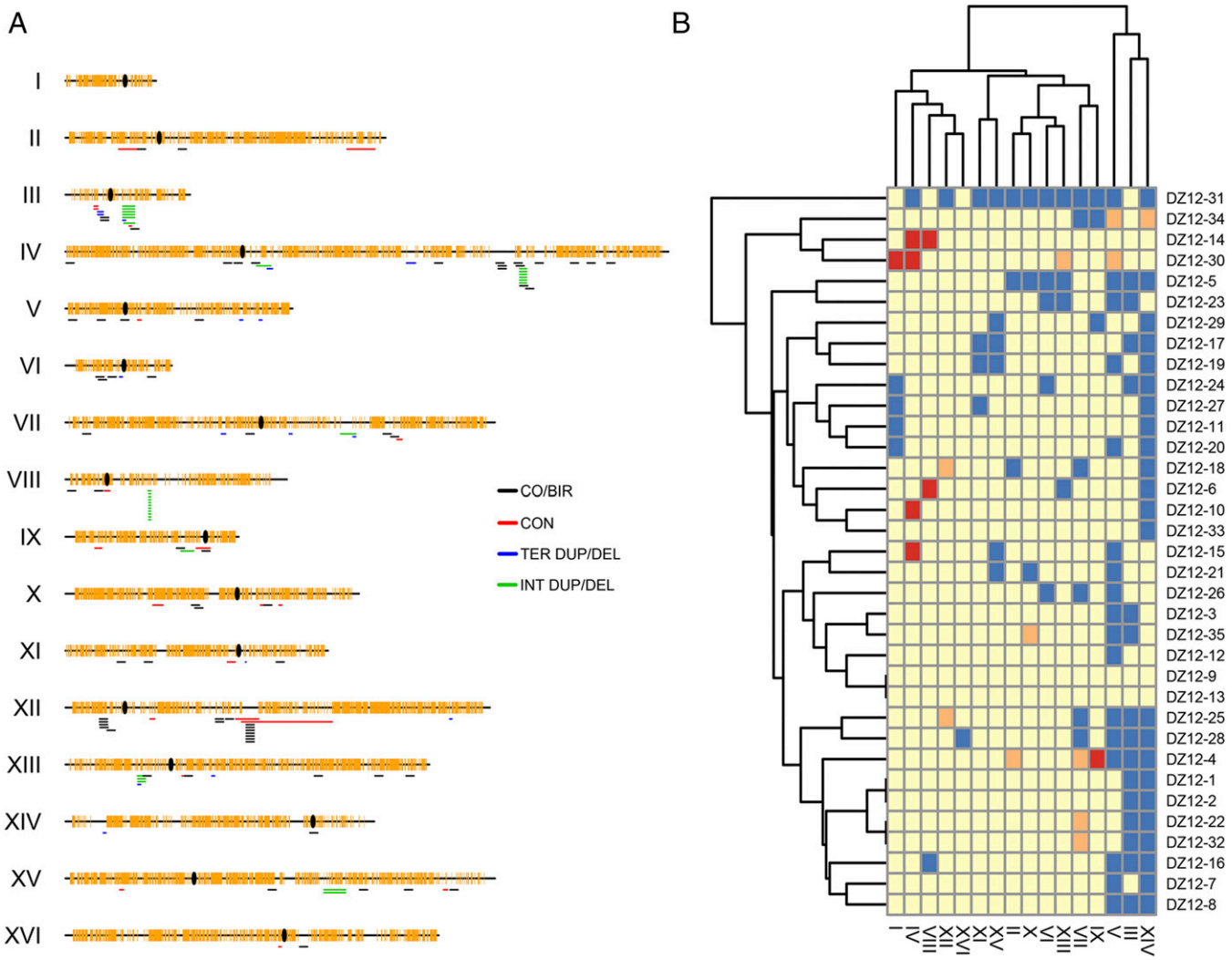

Fig. 2. Chromosomal alterations observed in 35 DZ12 derivatives. (*A*) The distribution of LOH and duplication/deletion events across yeast genome. CO/BIR, CON, TER DEL/DUP, and INT DUP/DEL represent crossover/BIR, gene conversion, terminal deletions/duplications, and interstitial duplications/deletions, respectively. Centromeres are shown as black ovals, and ORFs are shown as short vertical lines. (*B*) Two-way hierarchical clustering analysis of aneuploidy events. The red, orange, blue, and yellow colors indicate trisomy, uniparental disomy, monosomy, and no changes from euploidy, respectively.

low levels of two different components of the replisome result in similar levels of genomic instability.

Association of LOH Events with Elements of Chromosome Sequence or Structure. In yeast, certain motifs/sequences, including tRNA genes, Ty elements, LTRs, centromeres, DNA replication origins/terminations, G4 motifs, highly transcribed genes, and pause sites for Rrm3p are associated with slow-moving replication forks (*Supporting Information*). Longer pausing of replication forks increases the risk of fork collapse and DSBs. Phosphorylation of histone H2AX is an early response to DNA damage in eukaryotes, and regions of accumulated γ-H2AX were mapped as indicators of genomic fragile sites (18). Using a χ^2 analysis, we examined whether certain chromosomal elements were significantly overrepresented or underrepresented at the break points of genomic rearrangements.

We found four types of elements that were significantly overrepresented at recombination break points (Table S2): G4-/quadruplex-motifs, Ty elements, autonomously replicating sequence (ARS) elements, and γ-H2AX–enriched regions. The G4 motifs and the γ-H2AX–enriched regions are associated with slow-moving replication forks in wild-type cells, and the G4 motifs were overrepresented in our analysis of rearrangements induced by low polymerase α (10). In addition, several of the motifs that were significant in the low–polymerase-α dataset, such as replication-termination sequences, were nearly significant [uncorrected probability (*P*) value of 0.03] in strains with low polymerase δ. As an alternative method of comparing the two sets of data, we compared the ratio of observed events/expected events for the various classes of motifs (Fig. S4). The correlation coefficient was 0.75 and the *P* value was 0.002, confirming that replication stresses induced by low levels of two different DNA polymerases have shared properties.

Induction of Reciprocal Crossovers by Replication Stress. We previously developed a genetic system that allowed detection and mapping of reciprocal crossovers on the right arm of chromosome IV (19). Diploids for this purpose are homozygous for the ochre-suppressible *ade2-1* allele, and heterozygous for an insertion of the ochre-suppressing tRNA *SUP4-o* near the right telomere of chromosome IV. Strains with zero, one, and two copies of *SUP4-o* result in colonies that are red, pink, and white, respectively. A reciprocal crossover produces a red/white sectored colony (Fig. S5). To induce replication stress, we incubated DZ12 in medium with no galactose for 6 h, and then plated the culture in medium with high galactose. The frequency of red/white sectored colonies was 1.4×10^{-2} ($0.8–2.2 \times 10^{-2}$; 95% confidence limits). The frequency of sectored colonies in an isogenic wild-type strain is 3.1×10^{-5}. Thus, replication stress stimulates reciprocal crossovers about 500-fold.

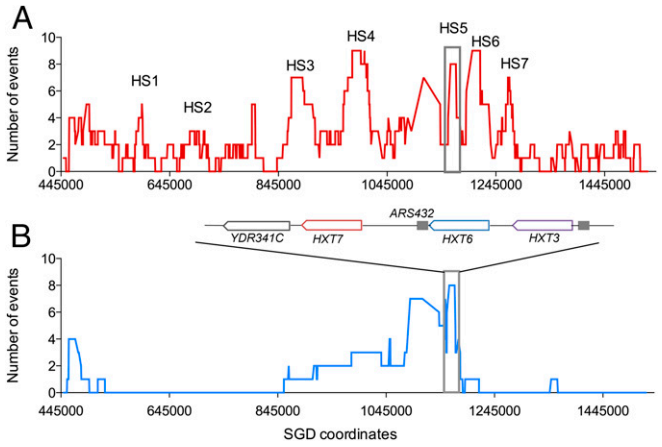

Fig. 3. Spontaneous recombination events and events induced by low levels of DNA polymerase δ on the right arm of chromosome IV. These plots summarize the number of times a SNP is included in conversion tracts associated with a crossover on chromosome IV. (*A*) Conversion tract distribution of spontaneous events (19). The labels HS1–HS7 are hot spots for crossovers. (*B*) Conversion tracts associated with low levels of DNA polymerase δ in DZ12. The highest peak contains the tandem array of the closely related *HXT7/6/3* genes.

Using SNP-specific microarrays, we mapped the transitions between heterozygous SNPs and homozygous ones. In addition to allowing us to map LOH associated with crossovers, the microarray analysis allows us to examine nonreciprocal LOH events (gene conversions) that are often associated with crossovers. From previous studies of spontaneous crossovers on chromosome IV, two classes of conversion are observed, those in which only one sister chromatid has a region of nonreciprocal LOH (Fig. S5*A*) and those in which two chromatids have such regions (Fig. S5*B*). The first class of event (3:1 conversions) is likely to reflect the repair of a single chromatid broken in S or G_2, whereas the second class (4:0 conversions) is likely to reflect the repair of two sister chromatids, resulting from replication of a chromosome broken in G_1. About two-thirds of the spontaneous crossovers in wild-type cells involve the repair of two sister chromatids (19).

The positions of the crossovers (SGD coordinates) in 34 red/white sectored colonies are in Dataset S7. Out of the 30 single-crossover events, 11 had no detected conversion tract (class A1, Dataset S8) and 19 have 3:1 conversion events (classes B1 and B2); 4 had two independent exchanges (class C). No 4:0 conversion tracts were observed. These results demonstrate that low levels of polymerase δ generate high levels of S/G_2-associated DNA lesions, as expected for cells experiencing replicative stress. Similar events were observed in cells with low levels of polymerase α (10). The median length of conversion tracts associated with crossovers in DZ12 was 7.1 kb (95% confidence limits of 5.1–19.8 kb), similar to spontaneous conversions (10.6 kb) and conversions induced by low polymerase α (6.3 kb).

St. Charles and Petes (19) previously identified several hot spots (HS1–HS7) for spontaneous crossovers on the right arm of chromosome IV (Fig. 3*A*). This distribution is clearly different from that induced by low levels of polymerase δ (Fig. 3*B*). The strong HS3 and HS4 spontaneous recombination hot spots were not prominent under replication stress. In contrast, the HS5 hot spot is enriched for recombination events in DZ12 ($P < 0.001$, by Fisher exact test). This region contains three closely linked repeated genes: *HXT7*, *HXT6*, and *HXT3* (Fig. 3*B*). In addition to the crossovers that map to this region, we observed six internal deletions between *HXT7* and *HXT6* within the sectored colonies (Dataset S9).

To examine what sequences/elements contribute to the HS5 hot-spot activity, we constructed diploid strains heterozygous for *URA3* and *HIS3* insertions flanking the hot spot (Fig. S6*A*). Five related diploids were generated: DZ67 (wild-type HS5 hot spot), DZ67d7 (deletion of *HXT7* with *hphMX4*), DZdA (deletion of *ARS432*), DZ67d6 (deletion of *HXT6*), and DZ67d3 (deletion of *HXT3*) (Fig. S6*B*). For all five strains, a crossover between *HIS3* and *URA3* will produce a cell that is 5-FOA^R His$^+$. As shown in Fig. S6*C*, deletion of either *HXT7* or *HXT3* resulted in a significant loss of hot-spot activity, whereas deletion of *ARS432* or *HXT6* led to small increases in recombination activity.

Genomic DNA Sequencing to Identify Small (<1-kb) Genomic Alterations in DZ12 Strains. To find alterations (point mutations and small in/dels) that would be undetectable by microarrays, we sequenced 15 out of the 35 DZ12-derived colonies grown on plates with low levels of galactose. We used Illumina paired-end high-throughput sequencing. All recombination events in the

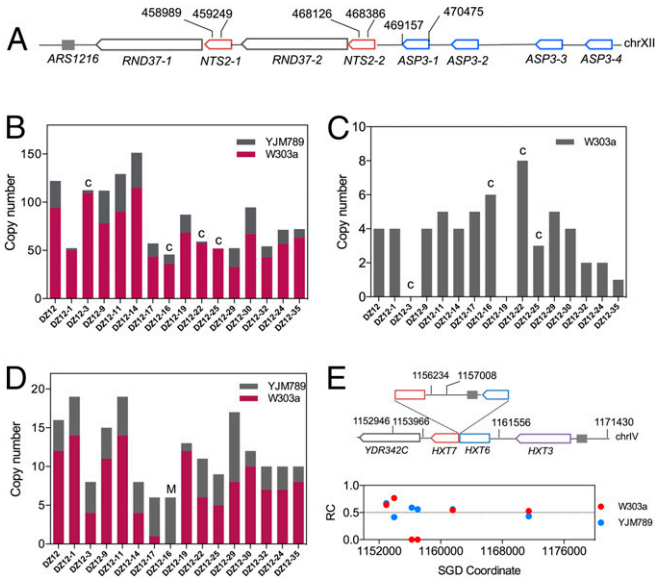

Fig. 4. CNV of repeated genes in strains with low levels of DNA polymerase δ. The copy numbers of each class of repeat were determined by DNA sequencing, using the number of "reads" for each repeat relative to the number of reads for single-copy sequences. (*A*) Depictions of ribosomal RNA and *ASP3* genes on chromosome XII; only 2 of the 150 copies of the rRNA genes are shown. The indicated SNPs in the nontranscribed spacer region were used to measure the copy numbers of W303-1A– and YJM789-derived rRNA genes. (*B*) Numbers of rRNA genes in 15 sequenced isolates compared with the parental strain DZ12. The gray and purple colors indicate YJM789- and W303-1A–derived repeats, respectively. In isolates labeled "C," there was a crossover within the rDNA. (*C*) The number of *ASP3* repeats in DZ12 and 15 isolates; this repeat is found only in the W303-1A–derived homolog. (*D*) The numbers of *CUP1* repeats. In the strain labeled "M," chromosome VIII (which contains the *CUP1* array) became monosomic. (*E*) An example of deletion between the *HXT7* and *HXT6* genes. This deletion was detected by a reduction in the sequencing coverage of the SNPs located at 1,156,234 and 1,157,008.

15 mutants previously detected by the microarray were confirmed by sequencing. Some findings obtained from the sequencing data are described below.

New LOH events. Although there are about 55,000 SNPs that distinguish the homologs of the hybrid strain, only about one-fourth of these SNPs (about 13,000) can be detected by microarray analysis (9). In our current analysis, in the 15 sequenced mutants, we detected 22 gene conversion events, of which 6 were already detected by microarray and 16 were new (Dataset S6). Most of the newly discovered conversion events had short tracts (median value of 1.7 kb; Dataset S6) compared with those detected by microarrays (Fig. S7). DNA sequencing also revealed that some conversion tracts were more complex than was evident from the microarray analysis (Fig. S8). In summary, although no additional crossovers or large (>5-kb) deletions or duplications were detected by sequencing, microarrays detected only about 30% of the gene conversion events.

Copy number variations in genomic regions with tandem repeats. Tandemly repeated genes represent about 10% of *Saccharomyces cerevisiae* genome (SGD: www.yeastgenome.org). Most of these repeated genes are not represented on our microarrays. By calculating the coverage of DNA sequencing reads from these regions, however, we were able to determine copy number changes for both the W303-1A– and YJM789-derived repeated genes.

The 9-kb ribosomal RNA genes form a cluster of about 150 tandem repeats on chromosome XII (20). In our analysis, we used four SNPs that distinguish the ribosomal DNA (rDNA) repeats of W303-1A from those of YJM789 (Fig. 4*A*). Coverage was determined by normalizing the number of "reads" for the repeated genes to the average number of reads for single-copy sequences (details in *Supporting Information*). The copy number of rDNA repeats in the starting DZ12 strain was about 122, of which 94 were derived from W303-1A and 28 were from YJM789 (Fig. 4*B*). The number of rDNA repeats per strain varied between 46 and 151 with a significant bias toward fewer repeats than the control strain. Because most of the isolates with altered numbers of repeats were not associated with a crossover between homologs, it is likely that the variation in copy number is a consequence of intrachromatid or sister chromatid recombination as will be discussed further below.

In some yeast strains, the centromere-distal end of the rDNA is adjacent to 3.6-kb repeats, each containing one copy of *ASP3* and a variant 5S rRNA gene (Fig. 4*A*, SGD website). From our analysis, we calculate that there are four copies of this repeat in the genome of W303-1A, but none in the YJM789 genome. Seven of the 15 isolates had an alteration in the number of repeats (Fig. 4*C*).

In a survey of 100 different *S. cerevisiae* strains, about two-thirds have tandem arrays of *CUP1* repeats (21, 22). Strain DZ12 has about 16 copies of *CUP1*, with 12 copies on the W303-1A–derived homolog and 4 on the YJM789 homolog (Fig. 4*D*). As observed for the rDNA, arrays in the 15 isolates were very frequently altered in size, and deletions outnumbered duplications.

The *HXT3*, *HXT6*, and *HXT7* genes are clustered as a tandem array on chromosome IV. *HXT6* and *HXT7* are almost identical (99.8% identity), whereas *HXT3* is 85% identical to *HXT6/7*. In the sectored colonies resulting from crossovers on chromosome IV, this cluster is a hot spot for LOH events (HS5 in Fig. 3*B*; Dataset S7) and for deletions that were associated with crossovers (Dataset S9). In addition, we found that 6 of the 35 unsectored colonies had deletions between *HXT6* and *HXT7*, resulting in a fusion of these two genes and loss of the intergenic region (Fig. 4*E*).

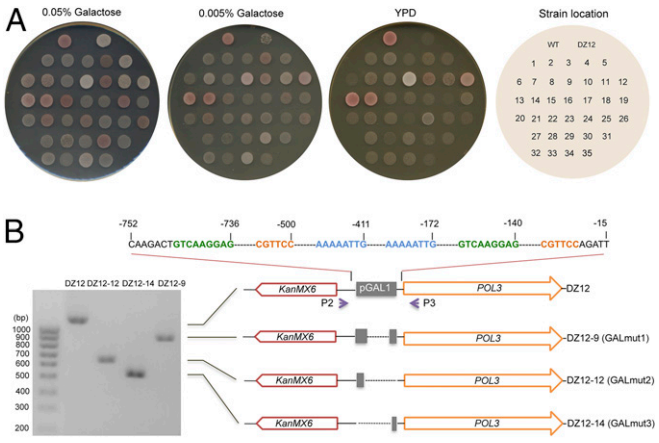

Fig. 5. DNA replication stress drives phenotypic changes in DZ12-derived isolates. (*A*) Comparison of the growth rates of a wild-type diploid (DZ3), DZ12, and 35 DZ12-derived isolates grown on medium containing low galactose. Approximately equal numbers of cells were placed on medium containing high [0.05% galactose, 3% (grams/100 milliliters) raffinose], low [0.005% galactose, 3% (grams/100 milliliters) raffinose], and YPD [2% (grams/100 milliliters) glucose, no galactose]. Numbers 1–35 represent the 35 DZ12-derived isolates (DZ12-1 to DZ12-35). (*B*) Deletions within the *GAL1* promoter in three DZ12 isolates capable of growth on YPD. When genomic samples of DZ12-12, DZ12-14, and DZ12-9 were amplified using primers P2 and P3 (Table S4), the resulting products were shorter than the original fragment derived from DZ12. By sequence analysis, we found that all three strains contained deletions that were flanked by microhomologies [shown in green (DZ12-14), orange (DZ12-12), and blue (DZ12-9)].

Single-base mutations and in/dels. Our microarrays do not detect single-base changes or most in/dels that are <1 kb. Among the 15 sequenced isolates, we found 71 single-base mutations (Dataset S10) and 11 in/dels (Dataset S11). Based on these numbers, the number of isolates, the number of cell divisions/isolate (about 25), and the diploid genome size, we calculated mutation rates for single-base mutations and in/dels as 8×10^{-9} and 1.3×10^{-9} per base per cell division, respectively. These rates are about 30-fold elevated for single-base mutations and about 500-fold elevated for in/dels compared with wild-type diploid strains (23, 24). A comparison of the point mutations with those observed in a wild-type strain is in Fig. S9. Most in/dels generated in DZ12-derivated isolates were deletions that ranged from 50 to 700 bp in length. One distinguishing characteristic of these deletions is the presence of two direct repeats (3–9 bp) flanking the deletion (Dataset S11).

Functional Consequences of Alterations Induced by Low Levels of DNA Polymerase δ. The genomes of solid metastatic tumors have many alterations (25), and, for the most part, it is unclear which of these alterations contribute to tumorigenicity. However, we found that some strains had alterations that allowed them to grow better under conditions of replication stress. As shown in Fig. 5*A*, DZ12-9, -12, -13, and -14 grew better than the starting DZ12 diploid on media containing low or no galactose (YPD). By sequencing DZ12-19, we identified a deletion in the *GAL-POL3* promoter (Fig. 5*B*). We used the primers P2 and P3 to examine the promoters of the other DZ12-derived colonies, and found two other isolates that grew well on YPD plates had deletions within the promoter; all three deletions were flanked by short repeats (Fig. 5*B*).

To verify the phenotypic effect of these three mutated promoters, we amplified the *kanMX6-PGAL-3HA* cassette from the strain without the promoter deletion and from the three deleted strains. These fragments were inserted into wild-type haploid derivatives of the W303-1A and YJM789. All transformants with the mutated *GAL1* promoters grew approximately as fast as the wild-type strains on low-galactose and YPD plates, whereas the strains with the unmutated promoter upstream of *POL3* cassette grew very slowly (Fig. S10).

In strains grown in the presence of galactose, transcription from the *GAL1* promoter is positively regulated by Gal4 (26) and, in the presence of glucose, is negatively regulated by Mig1 (27). The approximate locations of the Gal4 and Mig1 binding sites within the promoter are shown in Fig. S11. All three of the deletions that allow the *GAL1* promoter to function in medium containing glucose and lacking galactose remove the binding site for the Mig1 repressor. Although it is clear that removal of this region that negatively regulates transcription is important for the increased expression of *POL3*, the sequences/activators that positively influence expression are less clear. Two of the deletions partially or completely remove most of the sequences that define the *GAL1* promoter (Fig. S11). We note, however, a number of binding sites for Rtg1 and Gcr1 (transcriptional activators in the presence of glucose) located adjacent to the *GAL1* promoter (Fig. S11) that may positively influence transcription.

In Fig. 2*B*, we show that four of eight trisomy events involved chromosome IV, the chromosome encoding *POL3*. We also observed one large duplication event on the left arm of this chromosome that includes the *POL3* gene (DZ12-11). To determine whether an extra copy of *POL3* in these strains increases their ability to grow on low-galactose or YPD plates, we constructed derivatives of two of the trisomic strains (DZ12-10 and DZ12-30) in which we deleted one of the three *GAL-POL3* genes. We also deleted one of the *GAL-POL3* genes in the strain with a chromosome-borne duplication of *POL3* (DZ12-11). Strains with three *GAL-POL3* genes grew better than the derivatives with two copies (Fig. S12). Thus, strains that are trisomic for chromosome IV likely have a selective advantage when the cells are grown under low-galactose conditions, presumably because of increased levels of DNA polymerase δ.

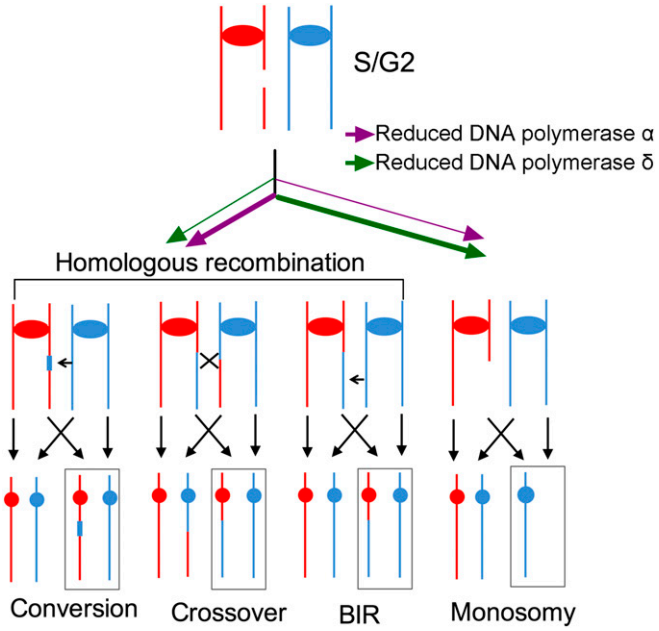

Fig. 6. Different patterns of chromosomal alterations caused by lowered expression of *POL1* and *POL3*. Both types of polymerase-depleted strains have elevated rates of DSBs during S and/or G_2. We suggest that these breaks are efficiently repaired in strains with low levels of DNA polymerase α, but inefficiently repaired in strains with low levels of DNA polymerase δ.

Discussion

Using SNP-specific microarrays and genome sequencing, we found that low levels of DNA polymerase δ greatly elevate numerous types of genomic instability including LOH events, aneuploidy, copy number variation (CNV), in/dels, and single-base changes. The level of instability in strains with low levels of DNA polymerase δ was similar to that observed in strains with low levels of DNA polymerase α, demonstrating that replication stress caused by deficiencies in two different components of the replisome can have similar quantitative effects. In addition, the genomic changes in strains with low levels of either DNA polymerase occurred preferentially in genomic regions that are associated with slow-moving replication forks. Last, we show that some of the genetic changes that occur in strains with low levels of polymerase δ relieve DNA replication stress. Below, we discuss the implications of these findings.

LOH and Aneuploidy. Strains with low DNA polymerase δ have rates of LOH that are about two orders of magnitude greater than observed in wild-type strains. Our mapping of crossovers on chromosome IV shows the location of the events is different in wild-type and low–polymerase-δ strains. The hot spots HS3 and HS4, each of which contains two closely linked inverted Ty elements (19), are absent in the low–polymerase-δ strain. In contrast, the HS5 hot spot that contains the tandemly repeated *HXT7*, *HXT6*, and *HXT3* genes is a preferred site for mitotic recombination in wild-type, low–polymerase-α and low–polymerase-δ strains. The same region had an elevated rate of interstitial deletions. One interpretation of this result is that template switching occurs between the *HXT* genes during DNA replication, forming secondary structures that can be resolved as DSBs resulting in either interhomolog crossovers or deletions. Alternatively, interstitial deletions could be formed by unequal crossovers or "pop-out" recombination (Fig. S13). Møller et al.

(28) found that the *HXT* repeats were also a source of extrachromosomal circular DNAs, presumably resulting from unequal intrachromatid crossovers between the repeats.

The DNA lesions that result in LOH in strains with low polymerase δ are different from those that induce LOH in wild-type strains in another important way. From the patterns of gene conversion associated with crossovers on chromosome IV (Fig. S5), we previously concluded that about two-thirds of the LOH events were initiated by DSBs induced in unreplicated chromosomes (19), whereas all of the conversion tracts observed in the present study reflect DSBs on only one chromatid. This result argues that the DNA breaks in strains with low levels of DNA polymerase δ occur in replicating/replicated DNA, likely as a consequence of a broken replication fork; similar results were observed in strains with low levels of DNA polymerase α (29). Our observation that LOH break points are enriched for hard-to-replicate sequence motifs is also consistent with this conclusion.

The rate of aneuploidy in strains with low levels of DNA polymerase δ was elevated more than two orders of magnitude compared with the wild-type strain. In addition, the ratio of aneuploid to LOH events is higher in strains with low levels of DNA polymerase δ than in strains with low levels of DNA polymerase α. One interpretation of this result is that DSBs in strains with low levels of DNA polymerase α are efficiently repaired (leading to more LOH events than chromosome loss events) relative to the efficiency of repair in strains with low levels of DNA polymerase δ (Fig. 6). This interpretation is consistent with the observations of Wang et al. (30), suggesting a greater requirement for DNA polymerase δ than α for the repair of HO-induced DSBs.

CNV. High rates of CNVs were observed in our experiments. Most of the interstitial deletions and duplications resulted from recombination between the *HXT* genes or nonallelic Ty elements, demonstrating that low levels of DNA polymerase greatly elevate the frequency of nonallelic homologous recombination events. Such events are associated with a large number of human diseases (31).

We also detected very frequent deletions in the rDNA and *CUP1* tandem arrays (Fig. 4). The frequent deletions can be interpreted in two ways. First, it is possible that the recombinogenic lesions that occur in the low-polymerase strains are channeled into a repair pathway (such as single-strand annealing) that preferentially produces deletions. Alternatively, strains with shorter arrays of rDNA and *CUP1* may have a selective growth advantage during replication stress. Previously, Kwan et al. (32) showed that the decreased use of rDNA replication origins resulted in elevated initiation activity for weak yeast origins. Thus, a reduction in the level of rDNA repeats (each of which contains an origin) may alleviate the replication stress imposed by the low level of DNA polymerase. It should also be noted that many of the rDNA origins are silenced during normal replication (33). Thus, under conditions of replication stress, if origin use is further compromised or if the rate of DNA fork movement is slowed, it is possible that strains with long arrays will have a selective disadvantage.

Small In/Dels and Single-Base Mutations. We previously found that strains with low polymerase δ had elevated rates of small deletions at the *CAN1* locus, and most of the deletions were flanked by short repeats (6). Similar mutations were observed in the current study (Dataset S11). Such mutations likely reflect DNA polymerase slippage (6). The alternative possibility that the deletions are a consequence of microhomology-mediated end joining is unlikely, because this class of events usually involve 8- to 10-bp flanking repeats (34).

We also found a 30-fold elevation in the rate of base substitutions. Northam et al. (35) showed a strong mutator phenotype associated with the *pol3-Y708A* allele. Most of these mutations were dependent on the error-prone DNA polymerase ζ. From these and other data, Northam et al. suggested that disruption of the normal replisome results in recruitment of DNA polymerase ζ even in the absence of DNA damage. One diagnostic type of mutation introduced by DNA polymerase ζ is a GC-to-CG alteration. In our analysis, this class of mutation was significantly ($P < 0.001$ by Fisher exact test) elevated in the low–polymerase-δ strain relative to wild type (Fig. S9). In summary, it is likely that the elevated rates of small in/dels and base substitutions in the low–DNA polymerase-δ strains reflect two different mechanisms: increased DNA polymerase slippage and recruitment of DNA polymerase ζ to slow-moving or stalled replication forks. It should be noted that DNA polymerase ζ does not increase the rate of in/dels involving short repeats (35). In addition, low levels of DNA polymerase α result in a very small (twofold) elevation in mutation rates (7).

Genetic Alterations That Relieve Replication Stress. The elevated rate of genomic alterations in strains with low DNA polymerase increases the likelihood of creating a strain with a genetic variant that could relieve replication stress. We found several examples of such variants. Three independent deletions within the promoter of the *kanMX6-GAL1-3xHA-POL3* cassette resulted in strains that grew better than the progenitor strain in low-galactose medium (Fig. 5). Second, we found that strains with an extra copy of the *kanMX6-GAL1-3xHA-POL3* cassette (acquired by chromosome nondisjunction or an intrachromosomal duplication) grew better on low-galactose medium. Last, we found that 23 of the 35 isolates examined by microarrays were monosomic for chromosome XIV. One possible explanation of this striking observation is that loss of one copy of XIV, which contains the genes encoding the catalytic subunits of DNA polymerases α and ε, helps preserve the stoichiometry of replication proteins in strains with low levels of DNA polymerase δ.

There is increased evidence that replication stress of various types can contribute to tumorigenesis (1). In our study, the stress induced by low levels of DNA polymerase δ results in greatly increased rates of multiple types of genetic alterations. It has been suggested that one approach to cancer therapy might be to apply additional conditions that further elevate stress, resulting in an intolerably high level of instability (36). In considering this approach, it is important to note how readily yeast strains with low levels of DNA polymerase δ produced variants that escaped replication stress.

Chromosome Instability Tumors. With the exception of tumors that have mutations in mismatch repair genes or the proofreading domains of DNA polymerases, most tumors do not have elevated rates of single-base or microsatellite mutations (11). Elevated rates of chromosomal changes (aneuploidy, CNV, LOH, and translocations), however, are common. Our current study and previous analysis of strains with low levels of DNA polymerase α (10) demonstrate that many features of this instability can be mimicked in yeast. Because null mutations in replicative DNA polymerases result in cell death, we suggest that hypomorphic alleles or epigenetic reductions in the levels of DNA polymerase or polymerase cofactors could be the initiating genetic alteration in a subset of chromosome instability tumors.

Experimental Procedures

Strains and Genetic Methods. The genotypes of yeast strains were given in Table S3. The details about strain construction are provided in *Supporting Information*, and the oligonucleotides used in constructions and analyses are in Table S4. Yeast transformations and matings were conducted using standard procedures. Analysis of genome stability using SNP-specific microarrays and the assay of recombination activity of the HS5 hot spot are described in *Supporting Information*.

Genome Sequencing and Analysis. Whole-genome sequencing of yeast strains was performed on the Illumina HiSeq 2500 sequencer using a 2 × 100-bp paired-end indexing protocol. The BWA software was used to align the high-quality reads to yeast genomic sequences (37). CNVs of tandem repeats (for example, rDNA) were determined by sequencing coverage. SNPs and small in/dels (<10-bp) detection were detected using Samtools (38) and VarScan (39). Deletions and duplications that are larger than 10 bp were detected by Lumpy (40).

Statistical Analysis. Most of the statistical tests followed the methods described in our previous studies (10, 41). Briefly, χ^2 tests were performed using VassarStat (vassarstats.net) or the chisq.test functions in Excel. Corrections of *P* values for multiple comparisons were performed as described by Hochberg and Benjamini (42). Fisher exact tests with two-tail *P* values and Pearson's correlation analysis were done using the software GraphPad Prism 6.

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